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Immunomodulatory effects of polysaccharides isolated from *Hericium erinaceus* on dendritic cells☆

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ABSTRACT

Hericium erinaceus (*H. erinaceus*; HE) polysaccharides (HE-PS) have been shown to have immunomodulatory activity. We found that the bioactive components of β-glucan derivatives consisted of 20% in HE-PS. We used an analytic platform for investigating the effects of HE-PS on the maturation of rat dendritic cells (DCs), which are derived from rat bone marrow hematopoietic cells (BMHCs). The results showed that treatment with 50 μg/mL HE-PS changed the morphology of the DCs to an active form in parallel with a significant two fold increase in MHC class II and CD80/86 surface antigens compared to the control. Furthermore, endocytosis by the DCs was significantly reduced at the same dosage. IL-12, IFN-γ and IL-10 cytokine secretion was significantly increased by 2.7, 1.5 and 1.6-fold, respectively, compared to the control after treatment with 50 μg/mL of HE-PS. This study used a powered analysis platform to show that HE-PS induces DCs activation and modulates the T_H1 immune response. Thus, HE-PS has potential as an immunopotentiating agent that could be further developed in the health food industry.

1. Introduction

Mushrooms are widely used in traditional Chinese medicine and have also been used as a food source for thousands of years. A number of mushroom species have been used as medicine or functional foods for the treatment of diseases in China, India, Japan, various other Asian countries and even the USA and Europe. Medicinal mushrooms were analyzed and found to contain several bioactive components, such as polysaccharides, bioactive proteins, glycoproteins, and proteoglycans, which can be antioxidants or antitumor factors [1–4].

Hericium erinaceus is a species of medicinal mushroom that has been used as a traditional Chinese medicinal fungus for many years. It is also called yamabushitake, houtou, the bearded hedgehog mushroom, the lion's mane mushroom, or the bearded tooth fungus in Japan, China, the USA and various other countries. It is used to treat gastric ulcers, chronic gastritis and other digestive tract-related diseases [5]. Previous studies have reported that both the fruiting bodies and the fungal mycelia contain bioactive polysaccharides that exhibit various pharmacological activities, including enhancement of the immune system, antitumor activity, hypoglycemic activity, anti-bacterial activity, anti-inflammatory properties, anti-aging properties and the acceleration of wound healing [6–17]. Specific compounds, such as threitol, D-arabinitol, and palmitic acid, are isolated from *H. erinaceus* and may have antioxidant effects as well as the ability to regulate lipid levels and to reduce blood glucose levels [13].

Many reports have demonstrated the immunomodulatory effects of polysaccharides [6–8,14–16]. The polysaccharides from *H. erinaceus*, which are larger than 1×10^5 kDa, have been shown to increase the levels of T cells and macrophages in mice [15]. They are also reported to induce the maturation of human DCs from human peripheral blood [8]. DCs are professional antigen-presenting cells that can interact with polysaccharides to bridge innate and adaptive immunity [18,19]. Upon activation, antigens are processed and presented by DCs that express high levels of costimulatory and major histocompatibility complex (MHC) molecules. DCs also secrete various cytokines and chemokines to modulate T and B lymphocyte responses. Furthermore, DCs are important mediators of peripheral immune tolerance and preservation of immune homeostasis [20].

Abbreviations: HE-PS, *Hericium erinaceus*-polysaccharides; DCs, dendritic cells; BMHCs, bone marrow hematopoietic cells; APCs, antigen presenting cells; TCA, trichloroacetic acid; LPS, lipopolysaccharide.

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In the present study, we used a well-established murine DC assay platform to measure the bioactivity of polysaccharides from *H. erinaceus*. The main goals were to evaluate the potential immunostimulatory effects of the polysaccharides by analyzing their effect on DC morphology, cytokine production, and endocytosis.

2. Materials and methods

2.1. Rats

Four week-old male CD (SD) IGS rats with initial weights between 76 g and 100 g were purchased from BioLasco (Taipei, Taiwan). Before the experiments, the rats were fed a basal diet with free access to water and feed for one week to adjust to the environment. The animal rooms were kept at 25 °C and 60% humidity with a 12 h light and dark cycle (8 AM–8 PM). All animal treatments were approved by the Animal Ethics Committee at the National Pingtung University of Science and Technology in Taiwan.

2.2. Preparation of crude HE-PS

Fruiting bodies of *H. erinaceus* were obtained from a local farm. The strain was purchased from Q-YO Bio-technology Co., Ltd. (Chunghwa, Taiwan). The cultivation temperature was 17 °C, and the relative humidity was 90%. The whole fruiting body was cleaned, homogenized and immersed in 97 °C ddH_2O for 4 h and then centrifuged at 10,000 × *g* for 10 min at 4 °C to yield a crude extract. Ethanol (95%) was used to precipitate the polysaccharides from the crude extract, and the polysaccharides were collected by centrifugation at 10,000 × *g* for 10 min at 4 °C. The crude polysaccharides were then lyophilized, ground and stored at −20 °C until use.

2.3. Measurement of the composition of HE-PS

The total sugar content of the extract was measured by a colorimetric method that has been previously described [21]. The total reducing sugars were analyzed using the 3,5-dinitro-salicylic acid method as previously described [22]. The absorbance of the sample solution was read at 540 nm. Glucose was used as a standard for both the total sugar content and the reducing sugar content analyses. An aniline blue assay was used to determine the concentration of β-1,3-D glucan in the extracts as previously described with some modifications [22,23]. Laminarin (Sigma, USA) was used as the β-1,3-D glucan standard. The protein concentration was determined by the Bradford method using a protein assay kit (Bio-Rad, USA) according to the manufacturer's instructions. Finally, the endotoxin content of the extracts was measured by a chromogenic *Limulus amedocyte* lysate kit (Associates of Cape Cod, USA) with a maximum sensitivity level of 0.25 EU/mL [6].

2.4. Determination of monosaccharide composition, properties and molecular weight (Mw) of the compounds in HE-PS

The identification and quantification of the monosaccharides of HE-PS was performed by gas chromatography (GC). First, the polysaccharides (6 mg) were hydrolyzed with 0.3 mL of 2 M trifluoroacetic acid (TFA) at 121 °C for 90 min. The hydrolyzed product was then reduced with $NaBH_4$ (2 g). This was followed by neutralization with dilute acetic acid and evaporation at 40 °C after the addition of 1 mg myoinositol as an internal standard and 1 M NH_4OH (0.1 mL) at 30 °C with stirring for 90 min. The reduced products (alditols) were acetylated and dried for GC analysis. By using this approach, the monosaccharides were conventionally converted into alditol acetates and analyzed by GC with 1 mg myo-inositol as the internal standard. GC was performed on a Hewlett-Packard HP 5890A Series II GC system (DE, USA) equipped with a DB-225 column (30 m × 0.25 mm × 0.15 μm). The column temperature was kept at 220 °C for 2 min and then increased to 250 °C for 3 min, and the compounds were separated at a rate of 1 mL/min for 30 min.

The Mw of the polysaccharides was determined by HPLC analysis. The crude polysaccharides were dissolved in 0.05 M NaCl (10 mg/mL), filtered through a 0.45 μm membrane and then applied as a 20 μL aliquot to the HPLC system (L-2490, Hitachi, Tokyo, Japan). The system was fitted with a TSK-GEL G3000PWXL column (7.8 mm × 30 cm) and was maintained at a temperature of 40 °C. The polysaccharides were eluted with 0.05 M NaCl solution at a flow rate of 0.6 mL/min and detected by a refractive index detector (RID). Dextran standards of various molecular weights (5900, 11,800, 22,800, 47,300, and 112,000 Da) were used to establish a standard curve for molecular weight determination.

2.5. Isolation of rat BMHCs and stimulation of BMHC-derived immature dendritic cells (BMHC-imDCs) with HE-PS

BMHCs were isolated from femurs of sacrificed rats (between 5 and 8 weeks old) as described previously [6,7]. On the sixth day of culture, the cells were collected and seeded into 24-well flat bottom culture plates (1×10^6 cells/well) with RPMI 1640 complete medium. The BMHC-imDCs had differentiated after 24 h of incubation (on the seventh day of culture), and the cells were then treated with various concentrations of HE-PS (0 as control, 3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) or 1 μg/mL lipopolysaccharides (LPS) for 48 h at 37 °C with 5% CO_2. Cell morphology, surface antigen presentation, cytokine secretion, and phagocytosis by the DCs were analyzed and compared to the untreated BMHC-imDCs.

Table 1
Composition of the crude extract of *H. erinaceus*.

Constituent	Content[a]	% of crude polysaccharide extract[a]
Total sugar	568 ± 45 mg/g	57 ± 5
Reducing sugar	46 ± 2 mg/g	5 ± 1
Protein	33 ± 1 μg/g	$3 \times 10^{-3} \pm 5 \times 10^{-6}$
β-1,3-D-Glucan	198 ± 65 mg/g	20 ± 7

[a] The values are shown as mean ± SD (n = 3).

2.6. Morphology of the BMHC-imDCs

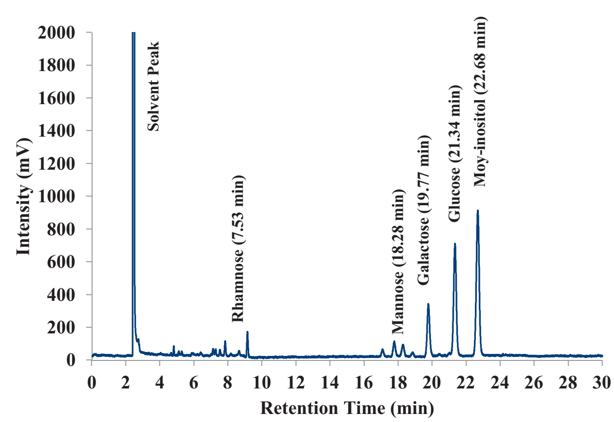
The morphology of the BMHCs and BMHC-imDCs was visualized with Liu's stain as described previously [6,7]. The cells were placed in a Cytospin (Thermo Scientific, UK), centrifuged at 350 × *g* for 10 min and stained with Liu's reagent. The morphology of the cells was observed by optical microscopy.

2.7. Measurement of BMHC-imDC surface markers by FACS analysis

The cells (1×10^6 cells/mL) that had been treated with or without HE-PS were collected, washed with FACS buffer (1% BSA and 0.1% sodium azide in PBS) and resuspended in 100 μL of FACS buffer. Fluorescent monoclonal antibodies (2 μL) against CD11c, MHC class II and CD80/86 surface antigens (eBioscience, USA) were mixed with the cells, and the mixture was incubated in the dark for 30 min. The cells were then treated with 0.5 mL of 3.8% paraformaldehyde. The labeled cells were analyzed on a FACScan flow cytometer (Becton Dickinson, USA). CellQuest Software was used to analyze the positively stained cells and to quantify the mean fluorescence intensity.

2.8. Surveying the endocytosis activity of the BMHC-imDCs

To measure BMHC-imDC endocytosis, 4 μL of 1 mg/mL FITC-dextran (Sigma) was added to 96 μL of 1×10^6 cells/mL (in PBS), and the cells were washed three times with PBS. Next, the cells were divided into two tubes, and one tube was incubated at 4 °C for 1 h as a background control, while the other tube was incubated at 37 °C for 1 h as a blank control. After 1 h, all the cells were washed three times with cold PBS (4 °C) and resuspended in 0.5 mL FACS buffer for analysis. The uptake of FITC-dextran was analyzed by cytofluorimetric analysis using a FACScan flow cytometer (Becton Dickinson, USA). CellQuest Software was used to analyze the cells with a positive reaction and to quantify the mean fluorescence intensity.



	Glucose	Galactose	Mannose	Rhamnose
Peak area	7553	3338.1	221.2	205.5
Molar ratio	36.8	16.2	1.1	1

Fig. 1. Composition of the *H. erinaceus* polysaccharides by GC analysis.

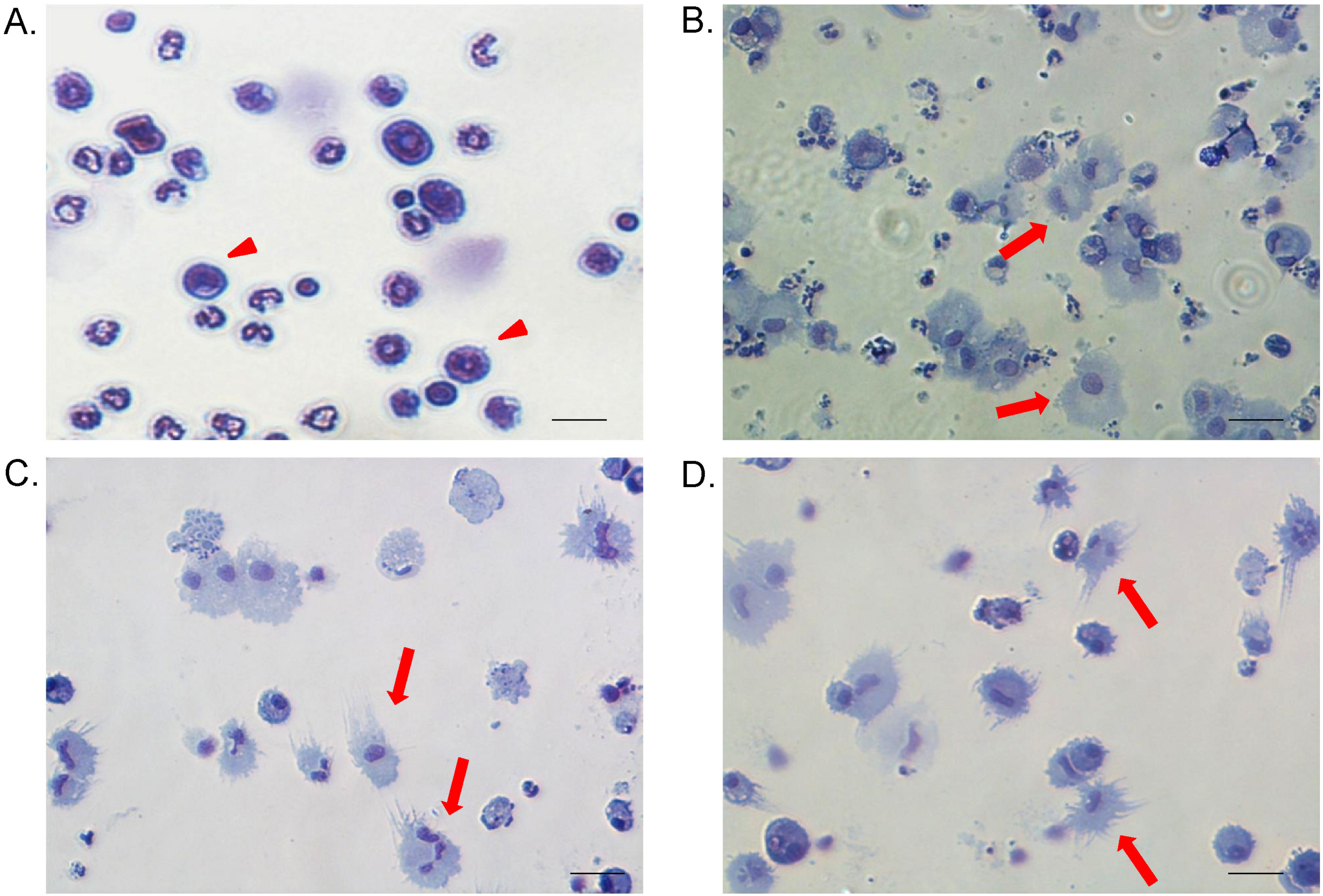


Fig. 2. Morphology of the dendritic cells after various treatments. (A) Morphology of BMHC isolated from rats (5–8 weeks old) at 200× magnification. (B) BMHC-imDCs after culture with 20 ng/mL rGM-CSF and 20 ng/mL rIL-4 for 7 days. (C) BMHC-imDCs after stimulation with 100 μg/mL HE-PS for 48 h. (D) BMHC-imDCs after stimulation with 1 μg/mL LPS for 48 h. The arrows indicate dendritic protrusions on the cell surface. The scale bars represent 50 μm.

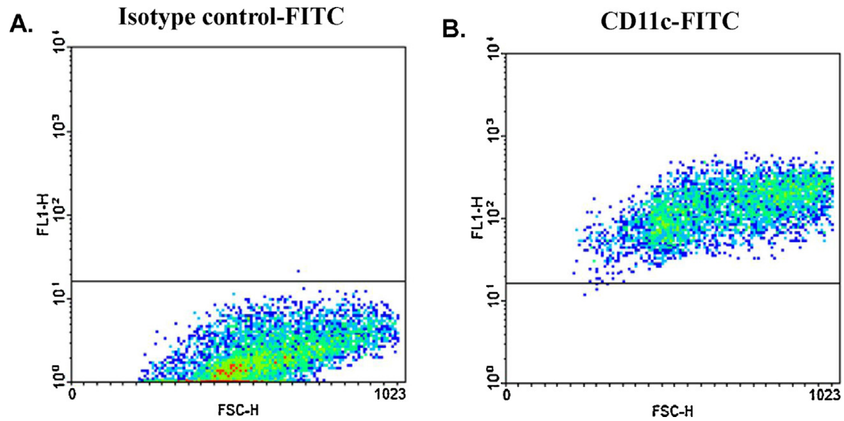


Fig. 3. Flow cytometric analysis of the CD11c cell surface marker on BMHC-imDCs after 7 days of culture with 20 ng/mL rGM-CSF and 20 ng/mL rIL-4. BMHC-imDCs were stained with either a FITC-conjugated isotype control or an anti-rat CD11c antibody.

2.9. Measurement of cytokine production by enzyme-linked immunosorbent assay (ELISA)

Various concentrations of HE-PS (0 as control, 3.125, 6.25, 12.5, 25, 50, and 100 μg/mL) were used to stimulate the BMHC-imDCs. After 48 hrs of treatment, the supernatants were collected by centrifugation at 400 × *g* for 8 min at 4 °C and were stored at −80 °C. An IL-12 p40 ELISA kit was used to determine IL-12 secretion according to the manufacturer's instructions (Biosource, USA), while IFN-γ and IL-10 expression was determined using a cytokine assay kit (Bender, Austria) according to the manufacturer's instructions.

2.10. Statistical analysis

The data were analyzed using Statistical Analysis System (SAS) software (SAS institute, USA) as described previously [6,7]. One-way analysis of variance (one-way ANOVA) and Duncan's test were used to determine the statistical significance between groups. Differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. Analysis of HE-PS

The fruiting bodies of *H. erinaceus* were extracted by hot water, and the polysaccharide was precipitated by alcohol to obtain a crude polysaccharide extract (HE-PS). The compositions of the crude HE-PS extracts were then analyzed. As shown in Table 1, the total sugar content was 568 mg/g (57% dry weight of the crude PS extract), while the reducing sugar content was 46 mg/g (5% dry weight of the crude PS extract). Furthermore, there were only trace amounts of protein (33 μg/g). As previously reported [22–25], β-1,3-D-glucan is a polysaccharide with known significant biological functionality. We determined that the β-1, 3-D-glycan content in the fruiting bodies of *H. erinaceus* was 198 mg/g (20% dry weight of crude PS extract) using laminarin as a standard for the aniline blue assay. The β-1,3-D-glucan content of *H. erinaceus* was much higher than that found in pullulanase hydrolyzed okra extract (0.6%) [6]. The monosaccharide composition of the crude extract from *H. erinaceus* was determined by GC analysis. The results showed that HE-PS was composed of rhamnose, mannose, galactose, and glucose in a molar ratio of 1:1.1:16.2:36.8, respectively (Fig. 1). These results differed from those of Jia et al. [26], though this might be due to a different growth environment of *H. erinaceus* [26]. Furthermore, six groups of polysaccharides with different molecular weights were separated and further purified by HPLC analysis (Suppl. Fig. 1A and B). The average molecular weights of the polysaccharides were approximately 2.2×10^4 Da. Polysaccharides with a molecular weight of 3.1×10^4 Da from *Lycium barbarum* have been shown to stimulate the maturation of murine bone marrow derived DCs [27]. The molecular size of HE-PS was similar to the *L. barbarum* polysaccharide, and we investigated whether HE-PS had the same immune-modulating effect on DCs in our study. Endotoxin contamination was surveyed prior to testing. The level of endotoxins was lower than 0.25 EU/mL in the HE-PS. These results show that any stimulatory effect of HE-PS was not due to the presence of endotoxins.

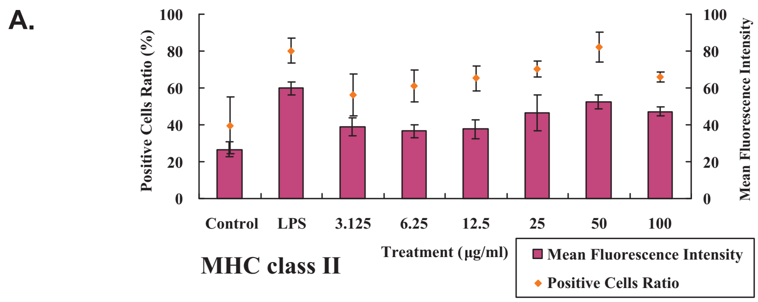
3.2. Morphological changes and maturation of BHMC-imDCs after HE-PS treatment

To measure the effect of HE-PS on BMHC-imDCs, the morphological changes in BMHC-imDCs were monitored after HE-PS treatment. Initially, live cell observation of the BMHC-imDCs was carried out. The BMHC-imDCs were obtained by treating BMHCs with rGM-CSF and rIL-4 for 9 days with or without 100 μg/mL of the HE-PS, which was added to the immature dendritic cells on the 7th day. The BMHC-imDCs were activated by the HE-PS, and dendritic protrusions could be seen on their cell surfaces (Suppl. Fig. 2A and B). These results are confirmed in Fig. 2 using Liu's stain, where the morphological changes to the DCs are clearly seen. In the absence of stimulation, the BHMC-imDCs were rounded with a polymorphic nucleus and small protrusions on the cell surface (Fig. 2A and B). After treatment with 100 μg/mL HE-PS (Fig. 2C) or 1 μg/mL LPS (stimulation control) for 48 h (Fig. 2D), the cells grew larger, and the nuclei became more polymorphic. Simultaneously, the cell surfaces of the dendritic protrusions became more pronounced and elongated. Our experiments strongly suggest that HE-PS treatment induced dendritic cell maturation.

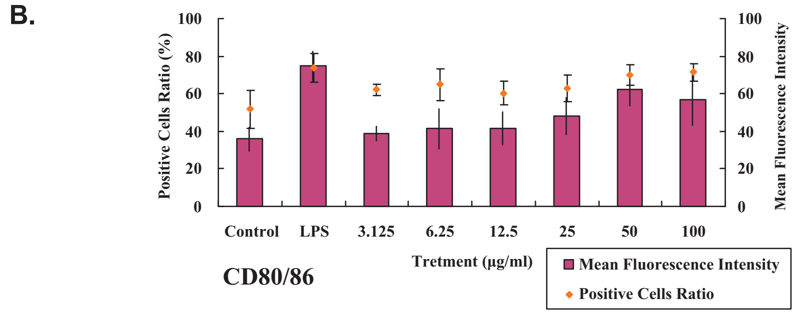
3.3. Expression of CD11c, MHC class II and CD80/86 by dendritic cells upon HE-PS activation

The cell marker CD11c (LFA-1) was used to identify the rat DCs [28–31]. After rGM-CSF and rIL-4 cytokine treatment, the BMHCs differentiated into BMHC-imDCs, which were approximately 91 ± 7% CD11c positive by FACS analysis (Fig. 3). To survey the activation of dendritic cells after treatment with HE-PS, CD80/86 and MHC class II expression levels were measured. During T cell activation, MHC class II is involved in antigen presentation, while CD80/CD86 are co-stimulatory molecules [32–34]. We used various concentrations of HE-PS (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) to treat the BMHC-imDCs for 48 h and induced them to mature. The positive cell ratio and the mean fluorescence intensity of MHC class II and CD80/86 was measured after treatment (Fig. 4).

Fig. 4A shows the results for MHC class II expression following HE-PS treatment at various concentrations compared to the LPS positive control and the untreated negative control, while Fig. 4B shows the results for CD80/86 expression. Our results indicate that



	Control	LPS	3.125	6.25	12.5	25	50	100
Positive cell ratio (%)	40 ± 15	80 ± 7	56 ± 11	61 ± 9	65 ± 7	70 ± 4	82 ± 8	66 ± 3
Mean of fluorescence intensity	27 ± 4	60 ± 4	39 ± 5	37 ± 4	38 ± 5	46 ± 10	52 ± 4	47 ± 3



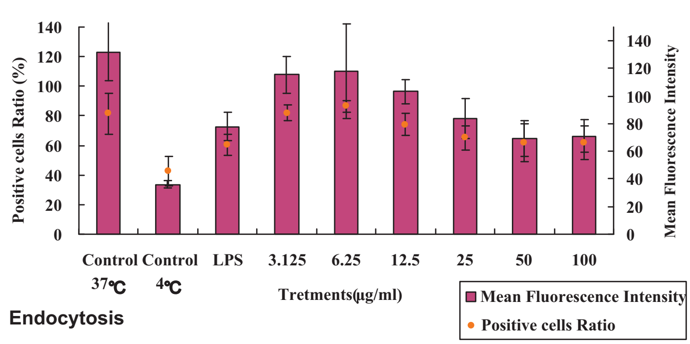
	Control	LPS	3.125	6.25	12.5	25	50	100
Positive cell ratio (%)	52 ± 10	74 ± 8	62 ± 3	65 ± 9	60 ± 6	63 ± 7	70 ± 6	71 ± 4
Mean of fluorescence intensity	36 ± 6	75 ± 8	39 ± 4	41 ± 11	42 ± 9	48 ± 10	62 ± 9	57 ± 13

Fig. 4. Expression of (A) MHC class II and (B) CD80/86 molecules on the surface of BMHC-imDCs stimulated with HE-PS (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) or LPS (1 μg/mL) for 48 hrs and then analyzed by flow cytometry. The positive cell ratio (%) and mean fluorescence intensity are shown as columns and dots, respectively.

treatment with HE-PS led to an increase in the positive cell ratio (82 ± 8% for MHC class II; 70 ± 6% for CD80/86) and the mean fluorescence intensity (52 ± 4% for MHC class II; 62 ± 9% for CD80/86) compared to the untreated control for both markers. The strongest activation of the DCs was observed at 50 μg/mL of HE-PS treatment (flow cytometry histogram in Suppl. Fig. 3). Therefore, HE-PS clearly enhanced the maturation of DCs.

3.4. Endocytosis by dendritic cells is decreased after HE-PS treatment

In the mammalian immune system, dendritic cells are antigen presenting cells that function by endocytosing, processing and presenting antigens to activate the adaptive immune system [35,36]. As previously reported, immature DCs are more endocytotic and



	Control 37℃	Control 4℃	LPS	3.125	6.25	12.5	25	50	100
Positive cell ratio (%)	87 ± 15	46 ± 10	65 ± 8	88 ± 6	93 ± 4	79 ± 8	70 ± 9	66 ± 14	66 ± 12
Mean of fluorescence intensity	123 ± 19	34 ± 2	73 ± 10	108 ± 12	110 ± 32	96 ± 8	78 ± 14	65 ± 12	66 ± 11

Fig. 5. Endocytosis of FITC-dextran by BMHC-imDCs stimulated with HE-PS (3.125, 6.25, 12.5, 25, 50 and 100 μg/mL) or LPS (1 μg/mL) for 48 h. The positive cell ratio (%) and mean fluorescence intensity are shown as columns and dots, respectively.

Table 2
Effect of different concentrations of HE-PS on the levels of IL-12, IL-10 and IFN-γ in DCs.

Treatments	Levels of cytokine[a] (pg/mL)		
	IL-12[b]	IFN-γ[b]	IL-10[b]
Control	216 ± 24[f]	90 ± 8[c]	53 ± 23[c]
LPS	742 ± 40[a]	169 ± 14[a]	148 ± 5[a]
HE-PS (μg/mL)			
3.125	362 ± 12[e]	108 ± 2[bc]	73 ± 5[bc]
6.25	368 ± 3[e]	108 ± 3[bc]	73 ± 16[bc]
12.5	424 ± 18[d]	106 ± 2[bc]	73 ± 6[bc]
25	445 ± 23[d]	117 ± 2[b]	71 ± 4[bc]
50	594 ± 13[c]	131 ± 17[b]	84 ± 4[b]
100	636 ± 17[b]	130 ± 14[b]	85 ± 4[b]

Data with different superscripts (a–f) in the same column are significantly different at $p < 0.05$.
[a] Levels of IL-12, IL-10 and IFN-γ in the supernatants were measured by ELISA.
[b] The values are mean ± S.D. and analyzed by one-way ANOVA; $n = 3$.

have greater processing activity than mature DCs. In contrast, mature DCs show lower endocytosis activity and higher antigen-presenting activity [37,38]. When endocytosis was measured by a FITC-dextran uptake assay after stimulation of BMHC-imDCs with various concentrations of HE-PS for 48 hrs, the positive cell ratio did not change significantly, but the mean fluorescence intensity was significantly reduced at 50 and 100 μg/mL HE-PS treatments compared to LPS (Fig. 5). The reduced endocytosis activity, as shown by the overall reduction in dextran uptake, suggests that HE-PS treatment leads to the activation of DCs.

3.5. HE-PS treatment of dendritic cells increases IL-12/IFN-γ secretion and reduces IL-10 secretion

Mature DCs display the peptide/MHC complex to CD4 T cells. DCs can also activate T cells via the secretion of cytokines, such as IL-12 and IFN-γ, which promotes the development of the T_H1 pathway, while the secretion of IL-10 favors the development of the T_H2 pathway [39]. As shown in Table 2, HE-PS significantly promoted the secretion of IL-12 in a dose-dependent manner. Upon treatment with 100 μg/mL of HE-PS, IL-12 secretion was enhanced by 2.9-fold compared to the negative control. Additionally, IFN-γ and IL-10 levels were significantly higher than the negative control when the HE-PS was used at 25 and 50 μg/mL, respectively. The production of IL-10 was slightly increased by 1.6-fold (Table 2). Taken together, the clear increases in IL-12/IFN-γ production by the DCs suggest that HE-PS may contribute to the T_H1 response.

4. Conclusions

This study demonstrates that HE-PS has an immunopotentiating effect on DCs. We have shown that HE-PS treatment promoted morphological changes of murine DCs and a reduction of DC endocytosis, which are consistent with DC maturation. Furthermore, HE-PS treatment also increased the expression of surface molecules that are important for antigen presentation. Finally, HE-PS treatment stimulated DCs to secrete cytokines that promote T_H1 responses. The above findings suggest that polysaccharides from *H. erinaceus* can play a role in functional foods as a polysaccharide adjuvant to aid immunomodulation and to enhance the immunogenicity of vaccines [40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.06.012.

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